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Single-Molecule Fluorescence Studies of Site-Specific DNA Recombination

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Site-specific DNA recombination plays a key role in bacterial cell division, viral life cycles and recombinant DNA technology. We present a general method for observing the action of recombinase proteins on surface-immobilised DNA molecules using single-molecule fluorescence. In conjunction with FRET we use a standard TIRF microscope with alternating laser excitation to monitor the intensity and point-spread-function width of the FRET acceptor, enabling us to interrogate the diffusive freedom of the fluorophore, which in turn reports on large scale conformational changes of DNA. This allows us to unambiguously assign each stage of the reaction and track its progress in real time on the slide surface.

We apply this assay to two recombinase proteins: Cre and Xer. First, we observe real-time recombination by the well characterized Cre recombinase, measuring FRET efficiencies that agree well with distances from the crystallised Cre-DNA complex. Furthermore we directly observe transitions occurring, at the 100ms timescale, between two isomers of the Holliday junction reaction intermediate, a key regulatory step in recombination.

We also investigate Xer, a more complex system involving the ATP-dependant translocase FtsK, where many mechanistic details are unclear. Our assay provides the first observations of synaptic complex formation, allowing us to directly investigate its stability and dependence on specific amino acids. Since there are no crystal structures of the Xer-DNA complex, our measurements provide the first glimpses of its structure and, intriguingly, we observe significant differences between Xer and Cre synaptic complexes.

Our assays permit observation of large-scale conformational changes of DNA, on the sub-second timescale, in parallel to the sensitive, small-scale measurements afforded by FRET, and allow us to study site-specific recombinases in unprecedented detail. The techniques are general and could be applied to other processes involving large scale DNA looping.

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Conformational Changes in MutS during Mismtach Repair Signaling Determined with Single Molecule FRET

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DNA mismatch repair (MMR) is required for high replication fidelity in organisms ranging from bacteria to humans. MutS protein initiates MMR by recognizing base-base mismatches and insertion-deletion mismatches in double stranded DNA. In spite of extensive study, a temporally resolved picture of MutS conformations during mismatch repair remains elusive. We used single molecule fluorescence resonance energy transfer (smFRET) to characterize conformational changes in Thermus aquaticus (Taq) MutS as it scans homoduplex DNA, recognizes mismatches, activates to a sliding clamp, and interacts with MutL. We found that DNA binding domains of MutS undergo large movements as it is converted to sliding clamp in a two step process. First, the proximate domains I, which are initially stabilized by interaction with a mismatch, partially open while MutS remains at the mismatch. The domains then move farther apart, which results in a ring conformation that allows MutS to slide on DNA. We also report interactions between MutS and MutL while bound to mismatched DNA. This information provides constraints for modeling the downstream MMR pathways.

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Real-Time Single Molecule Tethered Particle Motion Experiments Reveal the Kinetics and Mechanisms of Cre-Mediated Site Specific Recombination

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Tyrosine family recombinases (YRs) are widely utilized in genome engineering systems since they can easily direct DNA rearrangement. Cre recombinases, one of the most commonly used YRs, catalyze site-specific recombination between two *lox*P sites without a need for high-energy cofactors and other accessory proteins or a specific DNA target sequence between the *lox*P sites. Previous structural, analytical ultracentrifuge and electrophoretic analysis have provided the details on reaction kinetics and mechanisms for Cre recombinase, whether there are reaction intermediates or side-pathways is left unaddressed. Using tethered particle motion (TPM), the Cre-mediated site-specific recombination process has been delineated from the start to the end at the single molecule level, including the formation of abortive complexes and wayward complexes, serving as functional filters to prevent inactive nucleoprotein complexes from entering the recombination process. Reversibility and the formation of a thermal stable Holliday junction intermediate were observed within the Cre-mediated site-specific recombination process. Rate constants for each elementary step were determined, which explains the overall reaction outcomes under various conditions. Taken together, this study demonstrates the potential of the single-molecule methodology as an alternative approach to explore detailed reaction mechanisms.

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ATP-Induced Helicase Slippage Reveals Highly Coordinated Subunits

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Helicases are vital enzymes that carry out strand separation of duplex nucleic acids during replication, repair and recombination. Bacteriophage T7 gene product 4 is a model hexameric helicase that has been observed to use dTTP, but not ATP, to unwind dsDNA as it translocates from 5′ to 3′ along ssDNA. Whether and how different subunits of the helicase coordinate their chemomechanical activities and DNA binding during translocation is still under debate. Here we address this question using a single-molecule approach to monitor helicase unwinding. We found that T7 helicase does in fact unwind dsDNA in the presence of ATP and that the unwinding rate is even faster than that with dTTP. However, unwinding traces showed a remarkable sawtooth pattern where processive unwinding was repeatedly interrupted by sudden slippage events, ultimately preventing unwinding over a substantial distance. This behaviour was not observed with dTTP alone and was greatly reduced when ATP solution was supplemented with a small amount of dTTP. These findings presented an opportunity to use nucleotide mixtures to investigate helicase subunit coordination. We found that T7 helicase binds and hydrolyses ATP and dTTP by competitive kinetics such that the unwinding rate is dictated simply by their respective maximum rates Vmax, Michaelis constants Km and concentrations. In contrast, processivity does not follow a simple competitive behaviour and shows a cooperative dependence on nucleotide concentrations. This does not agree with an uncoordinated mechanism where each subunit functions independently, but supports a model where nearly all subunits coordinate their chemo-mechanical activities and DNA binding. Our data indicate that only one subunit at a time can accept a nucleotide while other subunits are nucleotide-ligated and they interact with the DNA to ensure processivity. Such coordination reveals a potential mechanism for regulation of DNA unwinding during replication.

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smFRET Experiments Reveal Conformational Changes in DNA Gyrase that Drive Strand Passage Towards Negative Supercoiling

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DNA topoisomerases catalyze the inter-conversion of DNA topoisomers and impact replication, recombination, and transcription. DNA gyrase negatively supercoils DNA in an ATP-dependent process. Supercoiling occurs by a strand passage mechanism that requires the opening and closing of three transient gyrase interfaces, the N-gate, the DNA-gate, and the C-gate. The mechanism of coordination of these conformational changes is currently unknown.

To dissect individual conformational changes and gain insight into their coordination, we monitored conformational changes of the gyrase DNA-gate, the N-gate, and the C-terminal domains (CTDs) of gyrase in single molecule FRET experiments with donor/acceptor labeled gyrase. In addition, we dissected the roles of DNA (G-segment) binding at the DNA-gate, wrapping by the CTDs, and capture of the transported DNA (T-segment) using linear DNAs of different lengths, and relaxed and negatively supercoiled plasmid DNA. We propose a detailed model for DNA supercoiling, in which the DNA bound at the DNA-gate is distorted and cleaved in a tightly coupled process. Flanking regions contact the CTDs, causing an upward movement. Upon complete wrapping of DNA, N-gate narrowing positions a T-segment in the upper cavity, and unlocks of the DNA-gate. ATP-binding then poises gyrase for strand passage: N-gate closure traps the T-segment, in a flip-flop mechanism triggers opening of the DNA-gate, and pushes the T-segment through the gap in the G-segment at the DNA-gate. DNA-gate closure renders strand passage irreversible, and the T-segment is released through the transiently opening C-gate. ATP hydrolysis, N-gate opening, and possibly CTD release complete the supercoiling cycle. Our results illustrate how a hierarchical, coordinated and tightly coupled sequence of conformational changes at the beginning of the supercoiling reaction strictly couples the nucleotide cycle to DNA strand passage in the catalytic cycle of gyrase.